Biochem. J. (1997) 323, 343–348 (Printed in Great Britain)

Selective inhibition of mitochondrial respiration and glycolysis in human leukaemic leucocytes by methylglyoxal

Swati BISWAS*, Manju RAY*§, Sanjoy MISRA†, D. P. DUTTA† and Subhankar RAY‡

*Department of Biological Chemistry, Indian Association for the Cultivation of Science, Calcutta 700 032, †Haematology Unit, N.R.S. Medical College and Hospital, Calcutta 700 014, and ‡Department of Biochemistry, University College of Science, University of Calcutta, Calcutta 700 019, India

The effect of methylglyoxal on the oxygen consumption of mitochondria of both normal and leukaemic leucocytes was tested by using different respiratory substrates and complex specific artificial electron donors and inhibitors. The results indicate that methylglyoxal strongly inhibits mitochondrial respiration in leukaemic leucocytes, whereas, at a much higher concentration, methylglyoxal fails to inhibit mitochondrial respiration in normal leucocytes. Methylglyoxal strongly inhibits ADP-stimulated α-oxoglutarate and malate plus NAD^+-dependent respiration, whereas, at a higher concentration, methylglyoxal fails to inhibit succinate and α-glycerophosphate-dependent respiration. Methylglyoxal also fails to inhibit respiration which is initiated by duroquinone and cannot inhibit oxygen consumption when the *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine by-pass is used. NADH oxidation by sub-mitochondrial particles of leukaemic leucocytes is also inhibited by methylglyoxal. Lactaldehyde, a catabolite of methylglyoxal, can exert a protective effect on the inhibition of leukaemic leucocyte mitochondrial respiration by methylglyoxal. Methylglyoxal also inhibits L-lactic acid formation by intact leukaemic leucocytes and critically reduces the ATP level of these cells, whereas methylglyoxal has no effect on normal leucocytes. We conclude that methylglyoxal inhibits glycolysis and the electron flow through mitochondrial complex I of leukaemic leucocytes. This is strikingly similar to our previous studies on mitochondrial respiration, glycolysis and ATP levels in Ehrlich ascites carcinoma cells [Ray, Dutta, Halder and Ray (1994) Biochem. J. **303**, 69–72; Halder, Ray and Ray (1993) Int. J. Cancer **54**, 443–449], which strongly suggests that the inhibition of electron flow through complex I of the mitochondrial respiratory chain and inhibition of glycolysis by methylglyoxal may be common characteristics of all malignant cells.

INTRODUCTION

Methylglyoxal is a normal metabolite with significant growth-inhibitory and anticancer properties (see [1] and the references cited therein). Recent work from our laboratory has clearly indicated that methylglyoxal is tumoricidal. It specifically inhibits respiration in a wide variety of malignant cells, whereas the respiration in normal cells remains unaffected under identical conditions of incubation [2]. By using a model malignant cell, the Ehrlich ascites carcinoma (EAC) cell developed in mice, we have further shown that methylglyoxal inhibits both mitochondrial respiration and glycolysis in this type of cells [3]. As a consequence of inhibition of both mitochondrial respiration and glycolysis, ATP levels in these cells have been found to be critically reduced, rendering the cells non-viable [3]. We have further observed that methylglyoxal specifically inhibits electron flow through complex I of the mitochondrial respiratory chain of EAC cells [4]. Interestingly, lactaldehyde, a catabolite of methylglyoxal, has been found to significantly protect both cellular [2] and mitochondrial [4] respiration in EAC cells. Methylglyoxal has also been found to inactivate glyceraldehyde-3-phosphate dehydrogenase (GA3PD, EC 1.2.1.12), a key enzyme of the glycolytic pathway, and this inactivation is responsible to a significant extent for the inhibition of glycolysis of EAC cells by methylglyoxal [3].

All the above-mentioned studies strongly suggest that in malignant cells both complex I and GA3PD, important components of the mitochondrial electron-transport chain and glycolysis respectively, are critically altered, and methylglyoxal acts at these altered sites. However, our previous studies were done with diverse types of normal and malignant tissue slices and cells. Because normal and leukaemic leucocytes are excellent comparable source materials to identify any possible alterations in the cellular metabolism of malignant cells, the present study was undertaken to investigate the possible inhibitory effect of methylglyoxal on mitochondrial respiration and glycolysis in normal and in a variety of leukaemic leucocytes. Moreover, the fact that the study has been carried out exclusively with human leucocytes has a profound clinical significance. The results of the present study clearly indicate an essential similarity between leukaemic leucocytes and EAC cells with respect to the effect of methylglyoxal.

MATERIALS AND METHODS

Chemicals

All the biochemicals were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Rotenone, malonate, antimycin A, duroquinone, 2-thenoyltrifluoroacetone (TTFA) and *N,N,N′N′*-tetramethyl-*p*-phenylenediamine (TMPD) were all products of Sigma. L-Lactaldehyde was prepared as described previously [4]. Commercial methylglyoxal was purified by vacuum distillation [5]. All other chemicals were of analytical grade and were obtained from local manufacturers.

Blood

Blood of leukaemic patients (suffering from chronic myeloid leukaemia or acute myeloid leukaemia, or chronic lymphocytic leukaemia or acute lymphocytic leukaemia) were collected from the haematology unit of the hospital. Patients with chronic myeloid leukaemia were selected for this study with their

Abbreviations used: EAC, Ehrlich ascites carcinoma; GA3PD, glyceraldehyde-3-phosphate dehydrogenase; α-GP, α-glycerophosphate; α-OG, α-oxoglutarate; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; TTFA, 2-thenoyltrifluoroacetone.

§ To whom correspondence should be addressed.

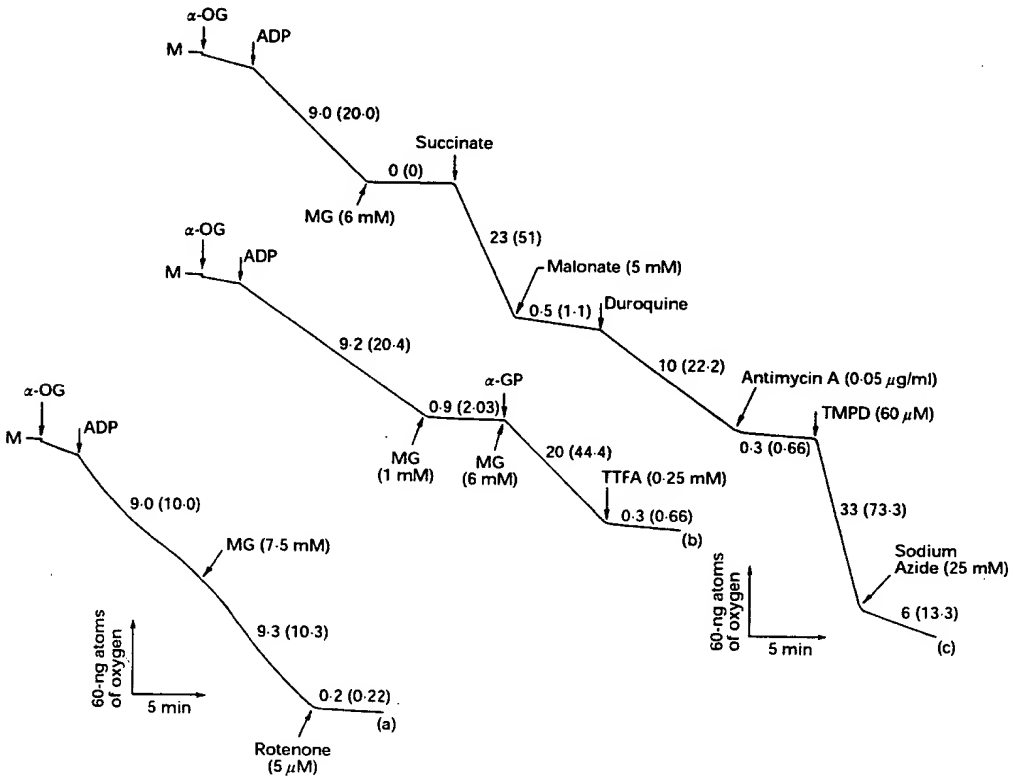

Figure 1 Effect of methylglyoxal on mitochondrial respiration in normal and leukaemic leucocytes

The direct oxygraph tracings of a typical experiment are shown. Trace (**a**) represents the effect of methylglyoxal on α-OG-dependent ADP-stimulated respiration of leucocyte mitochondria (0.9 mg of protein) from a healthy donor. Trace (**b**) represents the effect of methylglyoxal on ADP-stimulated mitochondrial (0.45 mg of protein) respiration in leukaemic leucocytes with α-OG and α-GP as substrates. Trace (**c**) shows the effect of methylglyoxal on ADP-stimulated respiration in leukaemic leucocyte mitochondria (0.45 mg of protein) with various substrates and inhibitors. Addition of methylglyoxal and other components is indicated in the respective tracings. The numbers without parentheses and the numbers within parentheses along the tracings represent the rate of oxygen consumption (ng-atoms of oxygen/min and ng-atom of oxygen/min per mg of protein respectively). In each case, in a control set of experiments, in which no methylglyoxal or inhibitor was added, mitochondria consumed oxygen at a linear rate for a period of 30 min at least with α-GP or succinate as substrate. With duroquinone or TMPD as substrate, the rate also remained almost linear for at least 15 min (results not shown). The total amount of oxygen present in the incubation medium was approx. 990 ng-atoms. Abbreviations: M, mitochondria; MG, methylglyoxal.

peripheral leucocyte count ranging from 90000 to 150000 cells/ml, where almost 95% of these cells were of leukaemic series. For other types of leukaemia, patients were selected with leucocyte counts of more than 25000 cells/ml. Normal blood was from healthy donors. For collection of blood, informed consent was obtained from the donors. The blood was brought to the laboratory at ambient temparature and the experiments were started within 1 h after withdrawal of blood.

For respiratory studies, blood was collected from 7 healthy male donors (21–45 years old) and 20 male and 7 female patients (9–55 years old). For the study of glycolysis, blood was collected from 5 healthy male donors (20–45 years old) and 6 male and 1 female patient (14–50 years old). We have observed that age and sex of donors have no influence on the experimental results. Results presented in this paper for leukaemic blood were obtained with mitochondria isolated form leucocytes of patients with chronic myeloid leukaemia. Smilar results were also obtained with mitochondria of other types of leukaemic leucocytes.

Preparation of mitochondria

Leucocytes were isolated from heparinized whole blood by gelatine treatment [6]. The leucocyte cell pellet was suspended in 5 vol. of 0.25 M sucrose, 10 mM potassium phosphate, 1 mM EDTA and 0.02% (w/v) BSA, pH finally adjusted to 7.2 with KOH. The cell suspension was homogenized in a tightly-fitting Potter–Elvehjem homogenizer with six up-and-down strokes. The homogenate was centrifuged at 800 *g* for 10 min and both the pellet and supernatant were retained separately. The pellet was suspended in 3 vol. of the above homogenizing buffer and homogenized in a Potter–Elvehjem homogenizer with three up-and-down strokes. The homogenate was centrifuged at 800 *g* for 10 min and the pellet was rejected. The supernatant was combined with the previous supernatant and centrifuged at 8000 *g* for 10 min. After rejecting the supernatant, the mitochondrial pellet was suspended in the above-mentioned buffer and washed twice by centrifugation at 8000 *g* for 10 min and again suspended in the same buffer for measurement of respiration and other studies.

Submitochondrial particles of leucocyte mitochondria were prepared by the method of Cain and Skilleter [7].

Measurement of respiration

Oxygen consumption was measured with an oxygraph (Gilson, Villiers-le-Bel, France) equipped with a clark electrode. The respiratory medium for the mitochondria of leucocytes con-

tained, in a total volume of 2.0 ml, 125 mM sucrose, 50 mM KCl, 5 mM Hepes buffer (pH 7.2), 2 mM KH_2PO_4, 1 mM $MgCl_2$ and respiratory substrates [10 mM α-oxoglutarate (α-OG) or 10 mM succinate or 10 mM α-glycerophosphate (α-GP) or 6 mM malate plus 1 mM NAD^+ or 10 mM glutamine or 10 mM glutamate or 5 mM duroquinone (dissolved in methanol)] and mitochondria containing 0.4–0.9 mg of protein. Other additions are mentioned in the Figure legends. The temperature of the incubation medium was 30 °C. After the indicated period of time, ADP (0.5 mM final concentration) was added to start phosphorylating respiration.

Estimation of metabolites and protein

Methylglyoxal [8], lactaldehyde [4], lactic acid [3], ATP [3], and protein with BSA as a standard [9] were estimated by the methods described in the respective references.

RESULTS

Figure 1 shows the effect of methylglyoxal on mitochondrial respiration in normal and leukaemic leucocytes with various respiratory substrates and inhibitors [10–12]. Methylglyoxal (7.5 mM) has no effect on α-OG-dependent ADP-stimulated mitochondrial respiration in normal leucocytes (trace a). However, this respiration can be readily inhibited by the complex I-specific inhibitor rotenone. In contrast, α-OG-dependent ADP-stimulated mitochondiral respiration in leukaemic leucocytes is almost completely inhibited with only 1 mM of methylglyoxal (trace b). Moreover, this methylglyoxal-inhibited respiration can be totally relieved by 10 mM α-GP, which after oxidation donates electrons directly to ubiquinone, which can be inhibited subsequently by TTFA. Results similar to those stated above were also obtained with malate plus NAD^+, glutamate and glutamine as respiratory substrate instead of α-OG (results not shown). That malate plus NAD^+ is a good substrate for leucocyte mitochondrial respiration had previously been shown by other investigators [13]. We have observed that the respiratory rate with only malate (6 mM) is very feeble, and is significantly increased in the presence of NAD^+ (1 mM). Both α-OG and malate plus NAD^+ donate electrons to complex I, whereas succinate donates electrons to complex II, and α-GP donates to ubiquinone, by-passing complex I. Methylglyoxal (6 mM) had no effect on mitochondrial respiration of leukaemic leucocytes when succinate and α-GP were used as respiratory substrate,

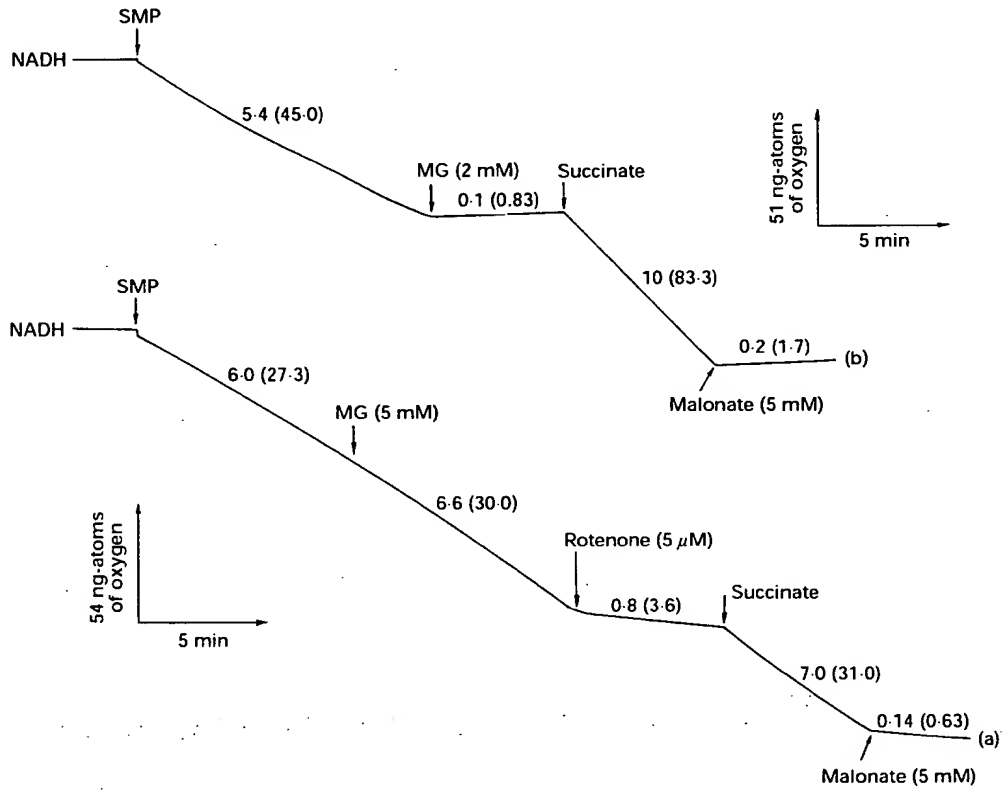

Figure 2 Effect of methylglyoxal (MG) on oxygen uptake by sub-mitochondrial particles (SMP) isolated from normal and leukaemic leucocytes with NADH or succinate as respiratory substrate

The direct oxygraph tracing of oxygen consumption by (a) SMP (0.22 mg of protein) and (b) SMP (0.12 mg of protein) prepared from normal leucocytes and leukaemic leucocytes respectively are shown. The details of the respiratory media are described in the Materials and methods section. Addition of methylglyoxal and other compounds are indicated on the traces. The numbers without parenthesis and the numbers within the parentheses along the traces represent the rate of oxygen consumption (ng-atoms of oxygen/min and ng-atoms of oxygen/min per mg of protein respectively). In a control set of experiments, where no methylglyoxal or inhibitor was added, the added SMP consumed oxygen at a linear rate for at least 30 min with NADH or succinate as the respiratory substrate (results not shown). The total amount of oxygen present in the mdeium was approx. 990 ng-atoms.

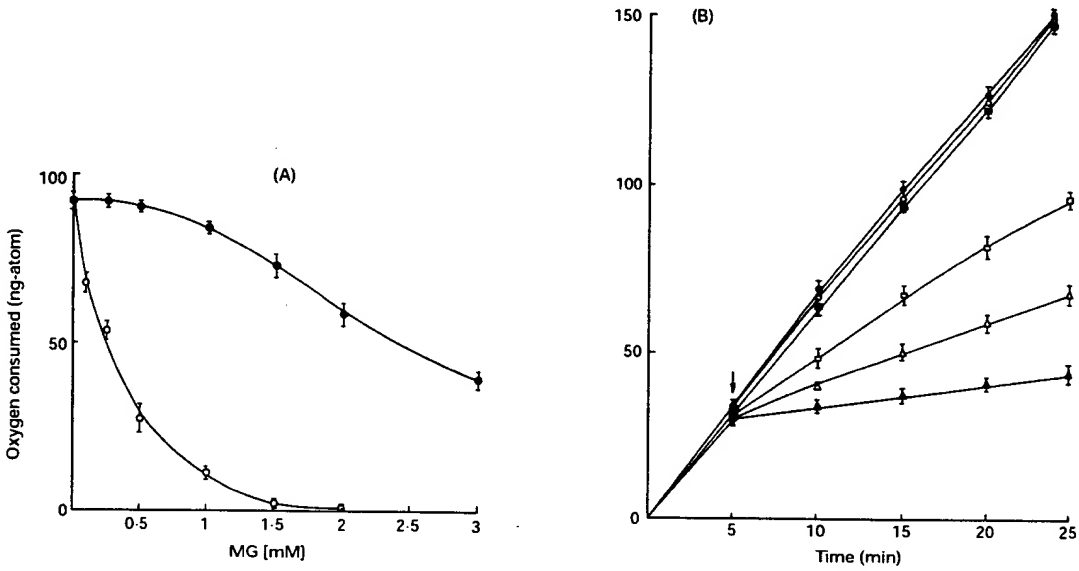

Figure 3 Protective effect of L-lactaldehyde on methylglyoxal (MG) inhibition of leukaemic leucocyte mitochondrial respiration with α-OG as respiratory substrate

(**A**) The effect of a fixed concentration (0.45 mM) of L-lactaldehyde on the inhibition of respiration with various concentrations of methylglyoxal (MG). (○) and (●) represent the oxygen consumption in the presence of various concentrations of methylglyoxal, but without lactaldehyde, and in presence of various concentrations of methylglyoxal and 0.45 mM lactaldehyde respectively. (**B**) The protective effect of different concentrations of lactaldehyde against the inhibitory effect of a fixed concentration of methylglyoxal on respiration, showing oxygen consumption: (○) in the absence of methylglyoxal or lactaldehyde; (●) in the presence of 0.5 mM lactaldehyde; and (△) and (▲) in the presence of 0.5 mM and 1 mM methylglyoxal respectively. □, Indicates the rate of oxygen consumption in the presence of 1 mM methylglyoxal and 0.15 mM lactaldehyde; and ■, in the presence of 1 mM methylglyoxal and 0.5 mM lactaldehyde. The arrow indicates addition of methylglyoxal. Mitochondria contained approx. 0.4–0.5 mg of protein. Each point represents the mean ± S.E.M. of four different samples from four different donors (3 male and 1 female). Experiments with each sample were performed in duplicate, and the average of two experiments from an individual donor was used to calculate the mean ± S.E.M. for the four donors.

which could be readily inhibited by malonate and TTFA respectively (results not shown). So, the results presented above strongly suggest that methylglyoxal inhibits electron flow through mitochondrial complex I of specifically leukaemic leucocytes.

Using α-OG as a respiratory substrate, we have studied mitochondrial respiration with 7 and 27 samples of normal and leukaemic leucocytes respectively. It has been observed that mitochondria of normal leucocytes consume 11 ± 3 ng-atoms of oxygen/mg per min, whereas leukaemic mitochondria consume 21 ± 5 ng-atom of oxygen/mg per min (values given ± S.E.M.). So it appears that leukaemic mitochondria are 1.8–2.5 times more active than their normal counterparts.

The effect of methylglyoxal on the specific complex of the mitochondrial respiratory chain of leukaemic leucocytes was further investigated by using several mitochondrial complex-specific inhibitors and electron donors. Trace (c) of Figure 1 shows again that methylglyoxal can readily inhibit α-OG-dependent, ADP-stimulated mitochondrial respiration in leukaemic leucocytes. Moreover, similarly to α-GP, succinate, an FAD-linked respiratory substrate which donates electrons to complex II, can readily relieve this inhibiton. Malonate, an inhibitor of succinic dehydrogenase, can inhibit this respiration, which re-commences when the complex III-specific artificial electron donor duroquinone is added to the system. Moreover, antimycin A, a complex III-specific inhibitor, inhibits this respiration, and the inhibition can be relieved by TMPD, which can donate electrons directly to complex IV after accepting them from other complexes. Trace (c) finally shows that this TMPD-dependent respiration can be blocked by sodium azide, a complex IV-specific inhibitor. All these studies confirm that the electron flow through complexes II, III and IV is not inhibited by methylglyoxal and the inhibitory effect of methylglyoxal on mitochondrial respiration in leukaemic leucocytes is due to inhibition of electron flow specifically through complex I.

That various metabolite transporters [14] are not involved in the inhibitory effect of methylglyoxal has been indicated by experiments with submitochondrial particles and by using different respiratory substrates.

From the results presented in this paper, it appears that methylglyoxal inhibits oxygen consumption in the mitochondria of leukaemic leucocytes with a variety of respiratory substrates, e.g. α-OG, malate plus NAD^+, glutamate and glutamine. All these substrates are NAD^+-linked and donate electrons to complex I. But these substrates are transported to mitochondria through some different, as well as some common, metabolite transporters [14]. On the other hand, the oxidation of succinate and α-GP, FAD-linked substrates which donate electrons, by-passing complex I, is not inhibited by methylglyoxal. It is unlikely that methylglyoxal is inhibitory to the transport of all the substrates which donate electrons to complex I, leaving unaffected the transport of succinate and α-GP, which donate electrons by-passing complex I. Succinate incidentally has a common transporter with malate.

The exclusive involvement of complex I in the inhibitory effect of methylglyoxal has been further substantiated by experiments using submitochondrial particles of leucocytes which are freely permeable to NADH. Metabolite transporters have no fuction in these preparations. Consequently, respiration in these particles is only limited by the dehydrogenase activity of the respiratory chain. Figure 2 shows that methylglyoxal can readily inhibit the

Table 1 Effect of methylglyoxal on L-lactic acid formation and ATP levels of leukocytes isolated from blood of leukaemic patients and healthy donors

A. Leukocytes (1.2×10^5 cells) from blood of patients with chronic myeloid leukaemia. B. Leukocytes (6×10^4 cells) from healthy donors. In both cases, the incubation mixture contained, in a total vol. of 1 ml, PBS (40 mM NaCl/2.7 mM KCl/6.5 mM Na_2HPO_4/1.5 mM KH_2PO_4, adjusted to pH 7.3 with NaOH), 5 μmol glucose and methylglyoxal as and where indicated. After 30 min of incubation at 37 °C in a metabolic shaker, the reaction was terminated with perchloric acid, and L-lactic acid and ATP were estimated. Control tubes were maintained with or without glucose and methylglyoxal as indicated, to correct for endogenous L-lactic acid. Addition of compounds just before termination of reactions is indicated by (*) which served as a zero-time control. Results are expressed per 10^5 cells. Values are means ± S.E.M. of five different samples from five different patients (4 male and 1 female) or five healthy donors (all male). Experiments with each sample were performed in duplicate, then the average of duplicate experiments from individual donors was used to calculate the mean ± S.E.M. for the five donors.

	Addition			
	Glucose (mM)	Methylglyoxal (mM)	L-Lactic acid formed (μmol/10^5 cells)	ATP level (nmols/10^5 cells)
A.				
	5	0	2.24 ± 0.076	153 ± 4.1
	5	2	0.59 ± 0.042	57 ± 3.0
	5	5	0.23 ± 0.55	50 ± 2.9
	5*	2	–	55 ± 3.5
	5*	5	–	45 ± 2.8
	5*	5*	–	98 ± 4.0
	0	0	–	96 ± 2.8
B.				
	5	0	0.77 ± 0.045	93 ± 3.7
	5	5	0.80 ± 0.03	88 ± 3.0
	5	10	0.77 ± 0.03	97 ± 3.6
	5*	5	–	65 ± 3.7
	5*	10	–	70 ± 3.2
	5*	5*	–	68 ± 3.8
	0	0	–	70 ± 3.0

oxidation of NADH by submitochondrial particles of leukaemic leucocytes, but it has no inhibitory effect on succinate oxidation, confirming that complex I is involved in methylglyoxal inhibition. Moreover, methylglyoxal has no inhibitory effect on NADH oxidation by submitochondrial particles of normal leucocytes.

We have previously reported that L-lactaldehyde, the product of enzymic reduction of methylglyoxal [15], could significantly protect the inhibitory effect of methylglyoxal on the viability [2] and mitochondrial respiration [4] of EAC cells. In the present study, by using leukaemic leucocyte mitochondria we have investigated the protective effect of lactaldehyde with various concentrations of lactaldehyde and methylglyoxal. Figure 3 shows that lactaldehyde exerts a strong protective effect against the inhibition of mitochondrial respiration by methylglyoxal. Mitochondrial respiration is almost 95% inhibited by 1 mM methylglyoxal. This inhibition is reduced to almost 50% and 0% in the presence of 0.15 mM and 0.5 mM L-lactaldehyde respectively.

We have previously observed that methylglyoxal inhibits glycolysis, as measured by L-lactic acid formation in EAC cells [3]. Because methylglyoxal inhibits mitochondrial respiration of EAC cells [3,4] and leukaemic leucocytes, the possible inhibitory effect of methylglyoxal on L-lactic acid formation and ATP levels in both normal and leukaemic leucocytes has also been investigated in the present work. Table 1 shows that methylglyoxal both strongly inhibits L-lactic acid formation and drastically reduces the level of ATP in leukaemic leucocytes only, whereas methylglyoxal has no effect on normal leucocytes.

DISCUSSION

Previous studies on glycolysis and respiration in intact leucocytes have indicated that both normal and leukaemic leukoytes have a high rate of aerobic glycolysis compared with other normal cells, although there is some variation with the type of leucocytes studied [16]. This observation has led to the suggestion that leucocytes derive most of their energy from glycolysis [17]. A search in the literature of leucocyte energy metabolism, however, indicates that the study of oxidative phosphorylation by leucocyte mitochondria has remained very scanty. In work on the oxidative phosphorylation by leucocyte mitochondria in blood of healthy humans, Foster and Terry [13], using leucocyte mitochondria, have demonstrated appreciable stimulation of endogenous respiration and ATP formation by the respiratory substrates succinate, α-GP and malate plus NAD^+. They concluded that leucocyte mitochondria are a mojor source of ATP [13].

The results presented here clearly show an appreciable amount of substrate-dependent, ADP-stimulated respiration by mitochondria of both normal and leukaemic leucocytes. Similarly to the work of Foster and Terry [13] we could use succinate, malate plus NAD^+ and α-GP as good respiratory substrates. Besides, α-OG has also been found to be a good substrate.

Our present study indicates that methylglyoxal specifically inhibits both mitochondrial respiration and glycolysis in leukaemic leucocytes, whereas it has no effect on the similar functions of normal leucocytes. Moreover, studies using several mitochondrial complex-specific substrates, artificial electron donors and inhibitors, and also the work with submitochondrial particles, clearly indicate that methylglyoxal specifically inhibits electron flow through complex I of malignant cell mitochondria. Because methylglyoxal inhibits both glycolysis and mitochondrial respiration it is expected that these effects would be reflected in the cellular ATP level. Table 1 clearly indicates that upon incubation of leukaemic leucocytes with methylglyoxal, the cellular ATP level is critically reduced.

The results presented in this paper on leukaemic leucocytes are strikingly similar to our previous studies on EAC cells [3,4], and it appears that the inhibitory effect of methylglyoxal on mitochondrial respiration and glycolysis may be a common feature of all the malignant cells. We have observed that methylglyoxal also specifically inactivates GA3PD in a wide variety of malignant cells (M. Ray, N. Basu and S. Ray, unpublished work). If pursued vigorously, there is a strong possibility that these studies may lead to the understanding of a serious biochemical difference between normal and malignant cells.

We (Ray and Ray) have recently proposed a new hypothesis on cancer, which suggests that excessive ATP formation in cells may lead to malignancy [18]. Methylglyoxal inhibits electron flow through complex I of the mitochondrial respiratory chain and inactivates GA3PD in malignant cells, which suggests that in malignant cells these are critically altered. In the cell, mitochondrial complex I is the principal component of ATP generation, and GA3PD is a component of glycolysis where ATP is generated. So, if the putative alterations at these two sites favour excessive ATP formation, then it is quite likely that this excess ATP will initiate all the anabolic reactions and the cell will grow, multiply and ultimately may turn malignant. The results presented in this paper and the discussions that follow strongly support the hypothesis.

This work was supported by grants (no. SP/SO/D11/93) from the Department of Science and Technology and the Council of Scientific and Industrial Research, Government of India. We thank Mr. Arup Bhaduri of N.R.S. Medical College and Hospital and Dr. Ajay K. Samanta of the Indian Institute of Chemical Biology, Calcutta, for their help in the collection of blood samples and cell counting.

REFERENCES

1 Misra, K., Banerjee, A. B, Ray, S. and Ray, M. (1996) Mol. Cell. Biochem. **156**, 117–124
2 Ray, M., Halder, J., Dutta, S. K. and Ray, S. (1991) Int. J. Cancer **47**, 603–609
3 Halder, J., Ray, M. and Ray, S. (1993) Int. J. Cancer **54**, 443–449
4 Ray, S., Dutta, S., Halder, J. and Ray, M. (1994) Biochem. J. **303**, 69–72
5 Argilés, J. M. (1989) Arch. Biochem. Biophys. **273**, 238–244
6 Schröder, J.-M., Mrowietz, U. and Christophers, E. (1988) J. Immunol. **140**, 3534–3540
7 Cain, K. and Skilleter, D. N. (1987) in Preparation and Use of Mitochondria in Toxicological Research, (Snell, K. and Mullock, B., eds.), pp. 217–254, IRL Press, Oxford
8 Cooper, R. A. (1975) Methods Enzymol. **41**, 502–508
9 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
10 Heisler, C. R. (1991) Biochem. Educ. **19**, 35–38
11 Hatefi, Y. (1985) Annu. Rev. Biochem. **54**, 1015–1069
12 Lee, C.-P., Sottocasa, G. L. and Ernster, L. (1967) Methods Enzymol. **10**, 33–37
13 Foster, J. M. and Terry, M. L. (1967) Blood **30**, 168–175
14 Klingenberg, M. (1979) Methods Enzymol. **56**, 245–252
15 Ray, M. and Ray, S. (1984) Biochim. Biophys. Acta **802**, 119–127
16 Seitz, I. F. (1965) Adv. Cancer Res. **9**, 303–410
17 Beck, W. S. (1958) J. Biol. Chem. **232**, 251–270
18 Ray, S. and Ray, M. (1997) Med. Hypotheses, in the press

Received 1 July 1996/21 November 1996; accepted 21 November 1996